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Bleached Pigment Activates Transduction in Salamander Cones

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ABSTRACT We have used suction electrode recording together with rapid steps into 0.5 mM IBMX solution to investigate changes in guanylyl cyclase velocity produced by pigment bleaching in isolated cones of the salamander *Ambystoma tigrinum*. Both backgrounds and bleaches accelerate the time course of current increase during steps into IBMX. We interpret this as evidence that the velocity of the guanylyl cyclase is increased in background light or after bleaching. Our results indicate that cyclase velocity increases nearly linearly with increasing percent pigment bleached but nonlinearly (and may saturate) with increasing background intensity. In cones (as previously demonstrated for rods), light-activated pigment and bleached pigment appear to have somewhat different effects on the transduction cascade. The effect of bleaching on cyclase rate is maintained for at least 15–20 min after the light is removed, much longer than is required after a bleach for circulating current and sensitivity to stabilize in an isolated cone. The effect on the cyclase rate can be completely reversed by treatment with liposomes containing 11-*cis* retinal. The effects of bleaching can also be partially reversed by β-ionone, an analogue of the chromophore 11-*cis*-retinal which does not form a covalent attachment to opsin. Perfusion of a bleached cone with β-ionone produces a rapid increase in circulating current and sensitivity, which rapidly reverses when the β-ionone is removed. Perfusion with β-ionone also causes a partial reversal of the bleach-induced acceleration of cyclase velocity. We conclude that bleaching produces an "equivalent background" excitation of the transduction cascade in cones, perhaps by a mechanism similar to that in rods.

INTRODUCTION

Exposure to light bright enough to bleach a significant fraction of the visual pigment produces a decrease in sensitivity which recovers slowly, as the pigment is regenerated. It is well known that, for scotopic vision (Campbell and Rushton, 1955) and for isolated rods (Cornwall, Fein, and MacNichol, 1990), the decrease in sensi-

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tivity after bleaching is much larger than can be accounted for by the decrease in the probability of light absorption by the photopigment. The most likely explanation is that bleached pigment produces an "equivalent background", which adapts like real light (Stiles and Crawford, 1932). Bleaching has been shown to activate both the guanylyl phosphodiesterase (PDE) and guanylyl cyclase in rods (Cornwall and Fain, 1994), probably by triggering the exchange of GDP for GTP on transducin in a manner similar to Rh* (Matthews, Cornwall, and Fain, 1994). Although the effectiveness of bleached pigment is only 10^{-7} to 10^{-6} that of Rh* (Cornwall and Fain, 1994), this appears to be sufficient after large bleaches to produce an "equivalent background" excitation strong enough to adapt the rod.

In cones, bleaching also produces desensitization larger than can be accounted for by the loss in quantum catch (Jones, Fein, MacNichol, and Cornwall, 1993), perhaps also as a result of an "equivalent background". Bleaches and backgrounds both produce a reduction in cone circulating current and an acceleration in the decay phase of the cone response (Baylor and Hodgkin, 1974; Matthews, Fain, Murphy, and Lamb, 1990; Jones et al., 1993). This suggests that bleached cone pigment can trigger transduction, though as yet there is no direct evidence for this.

We have used the method of rapid steps into IBMX solution (Hodgkin and Nunn, 1988; Cornwall and Fain, 1994) to show that bleached cone pigment activates the cone guanylyl cyclase, most probably by mimicking Rh* and triggering the transduction cascade. This "equivalent background" excitation is maintained for as long as bleached pigment is present but can be reversed by exposure to 11-*cis* retinal. It can also be partially reversed by perfusion with β-ionone, which has been shown to relieve bleaching desensitization even though this compound does not form a covalent attachment to the cone opsin (Jin, Crouch, Corson, Katz, MacNichol, and Cornwall, 1993).

These results have been reported in part at meetings of the Association for Research in Vision and Ophthalmology (Fain, Matthews, and Cornwall, 1993; Cornwall, Matthews, Crouch, and Fain, 1994*a*) and of the Physiological Society (Cornwall, Matthews, Crouch, and Fain, 1994*b*).

METHODS

Preparation and Recording

Larval tiger salamanders (*Ambystoma tigrinum*) were obtained from Charles Sullivan (Nashville, TN). Retinae were dissected and photoreceptors isolated as previously described (Matthews et al., 1990; Cornwall et al., 1990; Cornwall and Fain, 1994). Suction pipette recordings were made as in Cornwall et al. (1990), with the inner segment of the cone drawn into the pipette (Yau, McNaughton, and Hodgkin, 1981). All measurements were made from red-sensitive cones, identified by their greater sensitivity to long wavelengths (Attwell, Werblin, and Wilson, 1982; Perry and McNaughton, 1991). Suction pipette currents were filtered DC- 40 Hz with an active 8-pole Bessel filter and digitized at 250–1,000 Hz with Clampex of PCLAMP (Axon Instruments, Foster City, CA). All experiments were performed in Boston at an ambient (room) temperature of 20°C.

Solutions and Perfusion

Control saline solution contained (in millimolar): 104 NaCl, 2.5 KCl, 1 $CaCl_2$, 1.6 $MgCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.8 with ~8 mM NaOH. In some experiments, this solution

also contained 100 mg/liter bovine serum albumin. IBMX solution was made by adding 500 μM 3-isobutyl-1-methyl-xanthine (Sigma Chemical Co., St. Louis, MO) to the control saline. Solutions containing IBMX were made fresh the day of the experiment, and care was taken to add the IBMX to the same batch of control solution used for that experiment, to minimize the junction current.

The chamber containing the photoreceptors was continuously perfused with control saline. Cones were exposed to test solutions with a rapid microperfusion system as in Cornwall and Fain (1994). In some experiments, the microperfusion system was altered so that photoreceptors could be stepped into two or three rapidly flowing solutions instead of just one. Two or three inflow pipettes were fused adjacent to one another in a row, with their tips ~200 μm away from a single, opposed efflux pipette. Test solutions of different composition were perfused into all the inflow pipettes simultaneously, and the cone was then stepped into these solutions successively.

Light Stimulation, Bleaching, and Regeneration

Light stimuli were delivered as in Cornwall and Fain (1994). Absolute intensities of test and background beams were set at the beginning of each experiment with a diode photometer (model 80X, Graseby Optronics, Orlando, FL). Intensities in units of pigment molecules bleached per cone were calculated by assuming a collecting area for a salamander cone of 0.7 μm^2 for unpolarized light (Matthews et al., 1990; Perry and McNaughton, 1991).

In experiments in which we exposed isolated cones to bright bleaching light, we estimated the percentage of pigment bleached from the photosensitivity for vitamin A_2-based pigments in free solution (Dartnall, 1972), corrected for the difference in dichroism in free solution and in disk membranes (6.2×10^{-9} μm^2, G. Jones, personal communication). The validity of this method has been confirmed for isolated salamander cones by direct measurement of pigment bleaching in a microspectrophotometer (Jones et al., 1993).

Bleached pigment was regenerated by adding to the bathing medium liposomes containing 11-*cis* retinal, as previously described (Jin et al., 1993; Cornwall and Fain, 1994). The concentration of 11-*cis* retinal in the stock liposome solution was measured for each batch of liposomes in a spectrophotometer. The final concentration of 11-*cis* retinal in the bath was 10–20 μM.

The β-ionone was purchased from Sigma Chemicals (St. Louis, MO) and was repurified by double distillation. It was dissolved as a stock solution in EtOH and stored at −20°C. Aliquots from the stock solution were added to the control solution to give a final concentration of β-ionone of 5 μM (final EtOH concentration <0.1%). Cones were exposed to the β-ionone with the microperfusion system, which was altered for these experiments by replacing most of the plastic tubing with glass. The concentration of β-ionone at the outflow of the microperfusion system (i.e., at the point of entry into the bath) was measured with a spectrophotometer and was ~2 μM. When glass tubing was not used, the concentration of β-ionone was much lower and variable, probably because the β-ionone was absorbed by the walls of the plastic tubing.

Analysis of Data and Theory

Data were collected and analyzed as in Cornwall and Fain (1994), following a method introduced by Hodgkin and Nunn (1988). We have assumed that the light-sensitive current, j, is proportional to the free concentration of cGMP raised to a power N, for which we have taken a value of 3.0 (Yau and Baylor, 1989). As in Hodgkin and Nunn (1988), we let J represent the normalized value of the circulating current, that is $J = j/j_n$, where j_n is the circulating current in darkness. The cGMP economy of the cone can then be given by

$$\eta \frac{dJ^{\frac{1}{3}}}{dt} = \alpha' - \beta J^{\frac{1}{3}} \tag{1}$$

where η is the buffering capacity of the cone for cGMP and β is the velocity of the PDE. The parameter α' is proportional to the cyclase velocity (α) and is given by

$$\alpha' = \alpha \frac{[GTP]}{[cGMP]_n}, \tag{2}$$

where [GTP] is the concentration of GTP and $[cGMP]_n$ is a constant equal to the dark-adapted free cGMP concentration. Differences in the relative values of α' and α could occur if [GTP] changed during illumination, but this is likely to be a small effect (see Biernbaum and Bownds, 1985; Dawis, Graeff, Heyman, Walseth, and Goldberg, 1988; Apte, Ebry, and Dawson, 1993).

In the presence of steady background light or after a sufficiently long time after a bleach, the cyclase velocity will approach a steady value as the value of the circulating current and the intracellular Ca^{2+} concentration stabilize. The steady state rate of the cyclase can then be estimated by rapidly exposing cones to IBMX, which has been shown to block the PDE (Beavo, Rogers, Crofford, Hardman, Sutherland, and Newman, 1970). If the PDE is blocked with sufficient rapidity and completeness, the initial rate of current increase in IBMX should be determined by the steady state rate of synthesis of cGMP, since when $\beta[cGMP] \ll \alpha[GTP]$, then from Eq. 1,

$$\frac{dJ^{\frac{1}{3}}}{dt} = \frac{\alpha'}{\eta}. \tag{3}$$

Eq. 3 was used to estimate relative changes in α' from its value in darkness (Hodgkin and Nunn, 1988). The derivative of the one-third power of J was calculated with the program MATHCAD (MathSoft, Inc., Cambridge, MA) from $\Delta J^{1/3}/\Delta t$ centered upon t, with a Δt of 24 ms (because the currents were filtered at an f_c of 40 Hz). We have assumed η to be a constant, because at initial times when the peak value of the derivative was estimated, changes in [cGMP] would have been small.

There are three principal limitations of this method. First, our solution changes, though rapid (20–30 ms, see Cornwall, and Fain, 1994), were not instantaneous, and this is likely to have caused an underestimate of the cyclase velocity. Second, IBMX is a competitive inhibitor and, as such, produces a constant fractional suppression of PDE activity. Thus, the number of unblocked PDE's will increase as the total number of activated PDE's increases, i.e., with increasing background intensity or percent bleach. This may lead to a systematic decrease in the accuracy of the estimate of cyclase velocity with increasing light intensity or percent bleach. Third, the increase in current produced by blocking the PDE will most likely lead to an increase in Ca^{2+} flux into the cone, which will increase the Ca^{2+} concentration and produce a subsequent decrease in the velocity of the cyclase (Koch and Stryer, 1988). Thus, only the initial rate of current increase after the step into IBMX is useful, and even this initial rate may somewhat underestimate the extent of changes in cyclase rate.

RESULTS

Steps into IBMX: Backgrounds

As in previous experiments on rods (Cornwall and Fain, 1994), we have first attempted to estimate changes in the velocity of guanylyl cyclase in background light (Hodgkin and Nunn, 1988). A representative experiment from a single salamander cone in darkness and in three different background illuminations is given in Fig. 1. The current records to the left show the response produced by stepping the outer segment of the cone into 0.5 mM IBMX. The current was initially steady at the value corresponding to the intensity of the steady background. The step into IBMX

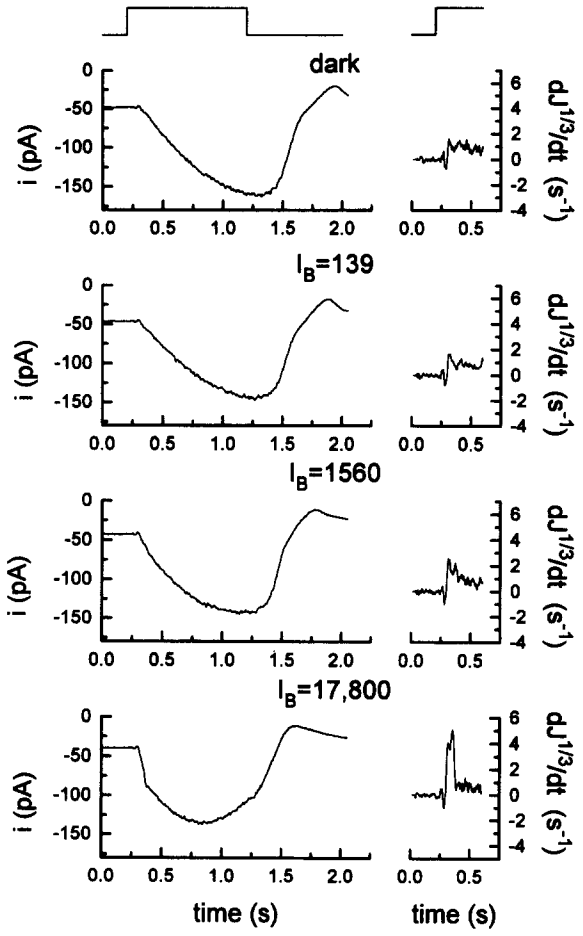

FIGURE 1. Steps into IBMX solution in darkness and in background light. (*Left*) Current responses to steps from control solution into 0.5 mM IBMX solution in darkness and in three backgrounds, as indicated (I_B = 139, I_B = 1,560, and I_B = 17,800) in units of photons μm^{-2} s^{-1} at 600 nm. Each current trace is the average of four measurements. (*Right*) Derivative of the one-third power of the normalized current for the first 0.6 s of the responses on the left. Uppermost traces give time course of triggers generated by PCLAMP software. The ~75 ms delay between the onset of the trigger and the beginning of the change in junction current was caused in part by a delay in the microprocessor that generates the signal controlling the stepping motor, coupled to the solution pipette assembly; and in part by the time required for the interface between the control and test solutions to reach the photoreceptor. Current responses have not been corrected for the small junction currents produced by addition of IBMX.

caused the current to increase, at a rate which was larger the brighter the background.

The derivatives at time t, calculated from the ratios $\Delta J^{1/3}\Delta t$ centered upon t, are given to the right in Fig. 1 for the initial part of the current record. They show a small negative-going excursion at the beginning of the IBMX step, probably due to a small junction current which we have not attempted to subtract. The derivatives then rise to a maximum and decline. A similar decline was seen in rods (Hodgkin and Nunn, 1988; Cobbs, 1991; Cornwall and Fain, 1994), where it is probably caused by the entry of Ca^{2+} through the light-dependent channels and inhibition of the cyclase. In cones, the decline in the derivative may be partially the result of the entry of Ca^{2+}, but a decline would also be expected from the increase in the cGMP-sensitive conductance and depolarization of membrane potential, due to the substantial slope conductance of the cGMP-sensitive channels (Attwell et al., 1982; Haynes and Yau, 1985). Because the derivative of $J^{1/3}$ is not constant during the IBMX step, we have estimated the relative value of the guanylyl cyclase velocity

from the maximum value of the derivative, which we abbreviate as δJ_{max} (Cornwall and Fain, 1994).

The value of δJ_{max} in darkness (δJ_{max}^{D}) ranged from 1.3 to 5.3 s^{-1} and averaged 2.5 ± 0.3 (SE, $n = 20$). This is larger than for rods, for which δJ_{max}^{D} averaged only ~1 s^{-1} (Cornwall and Fain, 1994). This would suggest that the dark velocity of the cyclase, like that of the PDE (Perry and McNaughton, 1991), is higher in cones than in rods. In Fig. 3 *A* we have plotted the ratio $\delta J_{max}/\delta J_{max}^{D}$ as a function of background intensity. As for rods (Cornwall and Fain, 1994), the increase in $\delta J_{max}/\delta J_{max}^{D}$ is nonlinear. To estimate the initial slope, we shall assume that for dim light δJ_{max} increases with the intensity of background light (I_B) according to

$$\delta J_{max} = \delta J_{max}^{D} + k_1 I_B, \tag{4}$$

where k_1 is a constant. We have fitted the open circles in Fig. 3 *A* for the first three backgrounds (139 to 1,560 photons $\mu m^{-2} s^{-1}$) with the equation,

$$\frac{\delta J_{max}}{\delta J_{max}^{D}} = 1 + \frac{k_1}{\delta J_{max}^{D}} I_B, \tag{5}$$

using a least-squares routine constrained to fit the point ($I_B = 0$, $\delta J_{max}/\delta J_{max}^{D} = 1$). This fitted curve is shown in the insert to Fig. 3 *A*. The best-fitting value of $k_1/\delta J_{max}^{D}$ was 3.4×10^{-4} photon^{-1} μm^2 s or $\sim 5 \times 10^{-4}$ $(Rh^*)^{-1}$ s. This is between one and two orders of magnitude lower than the analogous constant for salamander rods (2.2×10^{-2} $(Rh^*)^{-1}$ s, Cornwall and Fain, 1994), reflecting the higher values of δJ_{max}^{D} in cones and their lower sensitivity to light. At the brightest background where measurements were made (1.8×10^4 photons $\mu m^{-2} s^{-1}$), $\delta J_{max}/\delta J_{max}^{D}$ ranged from 1.8 to 5.8 and averaged 3.4 ± 0.6 (SE, $n = 7$).

Steps into IBMX: Bleaches

Bleaches also accelerate the rate of current increase during IBMX exposure. In Fig. 2, we show responses to IBMX steps from the same cone first in darkness, then after two representative bleaches, and finally after exposure to liposomes containing 11-*cis* retinal to regenerate the visual pigment. The current records are shown to the left and the time derivatives of $J^{1/3}$ for the initial part of the current record, to the right. Bleaches augment the initial rate of current increase, and this is reflected in the larger value of δJ_{max}. The bigger the bleach, the greater the increase in δJ_{max}. Exposure of 11-*cis* retinal brought δJ_{max} back nearly to its dark-adpated value.

In Fig. 3 *B*, we have summarized all of our measurements of the effect of bleaches on the rate of current increase in IBMX. The open squares give means with standard errors for $\delta J_{max}/\delta J_{max}^{D}$ as a function of percent bleach for seven cones. For each cone we regenerated with 11-*cis* retinal after all of the bleaches had been given, and the value of $\delta J_{max}/\delta J_{max}^{D}$ after regeneration is given as the filled circle. The data have been fitted with the equation

$$\frac{\delta J_{max}}{\delta J_{max}^{D}} = 1 + \frac{k_2}{\delta J_{max}^{D}} (\%\,bleach), \tag{6}$$

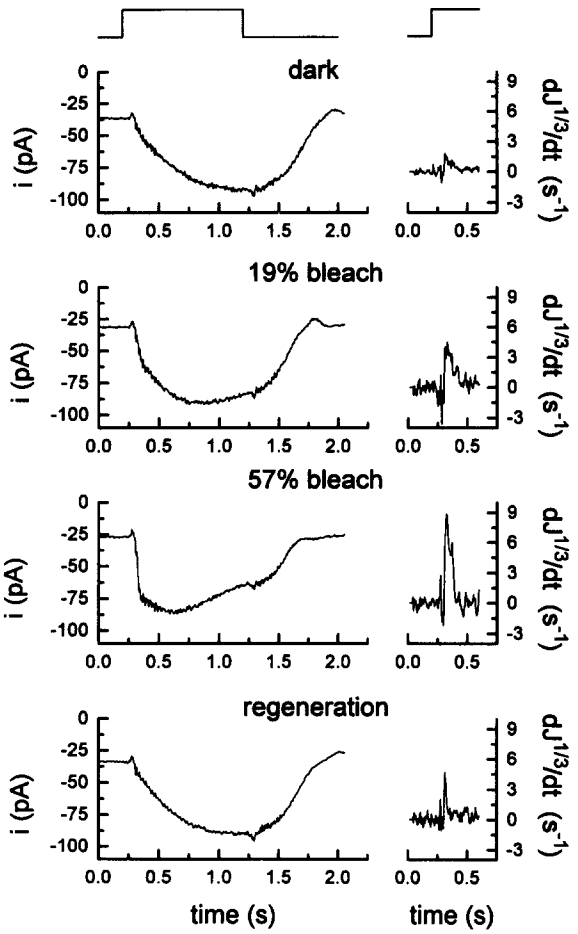

FIGURE 2. Steps into solution containing 500 μM IBMX in darkness, in steady state after exposure to bleaching light, and in steady state after regeneration with exogenous 11-*cis* retinal. (*Left*) Current responses to steps from control solution into IBMX under the following conditions (top to bottom): in darkness; 2–3 min after a total of 1 s exposure to a 600-nm light of intensity 3.4 × 10^7 photons $\mu m^{-2}s^{-1}$, which we estimate to have bleached 19% of the visual pigment; 2–3 min after a total of 4 s exposure to the same 600-nm light, which we estimate to have increased the percentage of pigment bleached to 57%; and 10–11 min after exposure to liposomes containing 11-*cis* retinal (see Methods). Each response is the average of four measurements. (*Right*) Derivative of the one-third power of the normalized current for the first 0.6 s of the responses on the left. Uppermost traces give time course of trigger pulses generated by the PCLAMP software (see Fig. 1).

with a best-fitting value for $k_2/\delta J_{max}^D$ of 0.026 (percent of bleach)$^{-1}$. Our rationale for fitting these data with a straight line is as follows. We assume that for cones, as for rods (Cornwall and Fain, 1994), PDE rate increases proportionally with percent bleach, because each bleached pigment molecule would be expected to activate the transduction cascade with the same probability. If the PDE rate is linear with percent bleach, the cyclase rate should also be nearly linear, since at steady state $dJ/dt = 0$, and from Eq. 1,

$$\frac{\alpha'}{\alpha'^D} = \frac{\beta}{\beta^D}\left(\frac{j}{j^n}\right)^{\frac{1}{3}} \tag{7}$$

Even for the brightest bleach $(j/j_n)^{1/3}$ never fell below ~0.9.

Assuming that the volume of a cone outer segment is 70 μm^3 (Matthews et al., 1990) and that the concentration of pigment in a cone is the same as in a rod (3 mM,

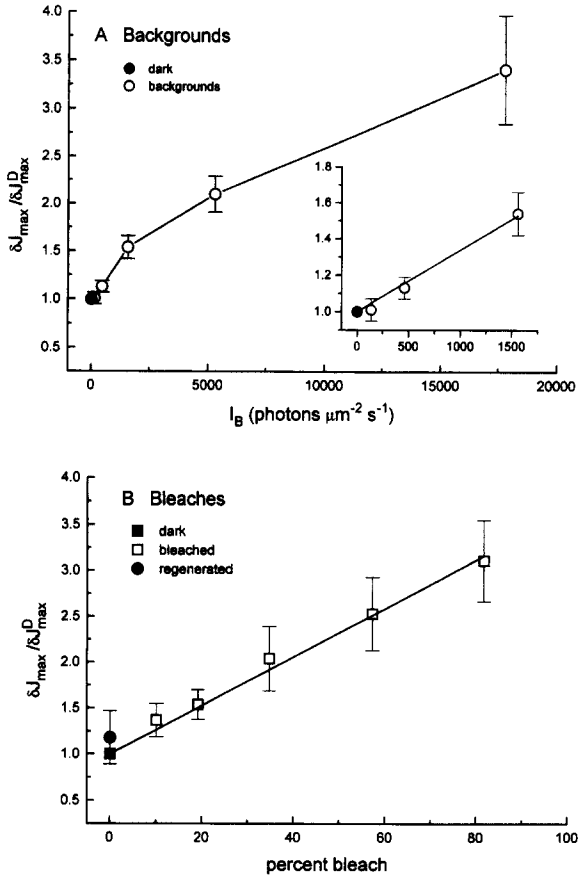

FIGURE 3. Dependence of $\delta J_{max}/\delta J^D_{max}$ on background intensity and percent pigment bleached. Values for δJ_{max} and δJ^D_{max} were obtained for each cell as in Figs. 1 and 2 from the one-third power of the normalized current after stepping into control solution containing 500 μM IBMX. (*A*) Means ± SE's of $\delta J_{max}/\delta J^D_{max}$ for seven cones in darkness (●) and in 600 nm background light (○). (*Insert*) $\delta J_{max}/\delta J^D_{max}$ in darkness and for the three dimmest backgrounds replotted on expanded axes. Straight line is linear regression constrained to pass through the point (I_B = 0, $\delta J_{max}/\delta J^D_{max}$ = 1). See text. (*B*) Means ± SE's of $\delta J_{max}/\delta J^D_{max}$ for seven cones in darkness (■), after a series of bleaches of increasing duration (□), and after regeneration of the photopigment with 11- *cis* retinal (●). Straight line is linear regression constrained to pass through the point (percent bleach = 0, $\delta J_{max}/\delta J^D_{max}$ = 1). See text.

Liebman, 1972), we calculate that there are ~1.3×10^8 pigment molecules per cone outer segment. Thus, a 1% bleach is equivalent to 1.3×10^6 bleached pigment molecules (Op), and $k_2/\delta J^D_{max}$ can be given as 2.0×10^{-8} Op^{-1}, ~20-fold larger than the equivalent value for rods (1.2×10^{-9} Op^{-1}, Cornwall and Fain, 1994).

Exposure of Bleached Cones to β-Ionone

The effects of bleaching on circulating current, sensitivity, and time course of response can be partially reversed by treatment with β-ionone, which has a cyclohexenyl ring similar to 11-*cis*-retinal but with a truncated polyene chain (Jin et al., 1993). β-ionone can be put directly in the control solution and can therefore be delivered through the rapid perfusion system (see Methods). An experiment showing the effects of rapid exposure to β-ionone on a bleached cone is given in Fig. 4. The cone in this figure had a dark circulating current of 35.7 pA. After illumination with a light calculated to bleach ~82% of the visual pigment, the cone was rapidly exposed to 2 μM β-ionone (Fig. 4 *A*, *first arrow*). This produced a negative deflection

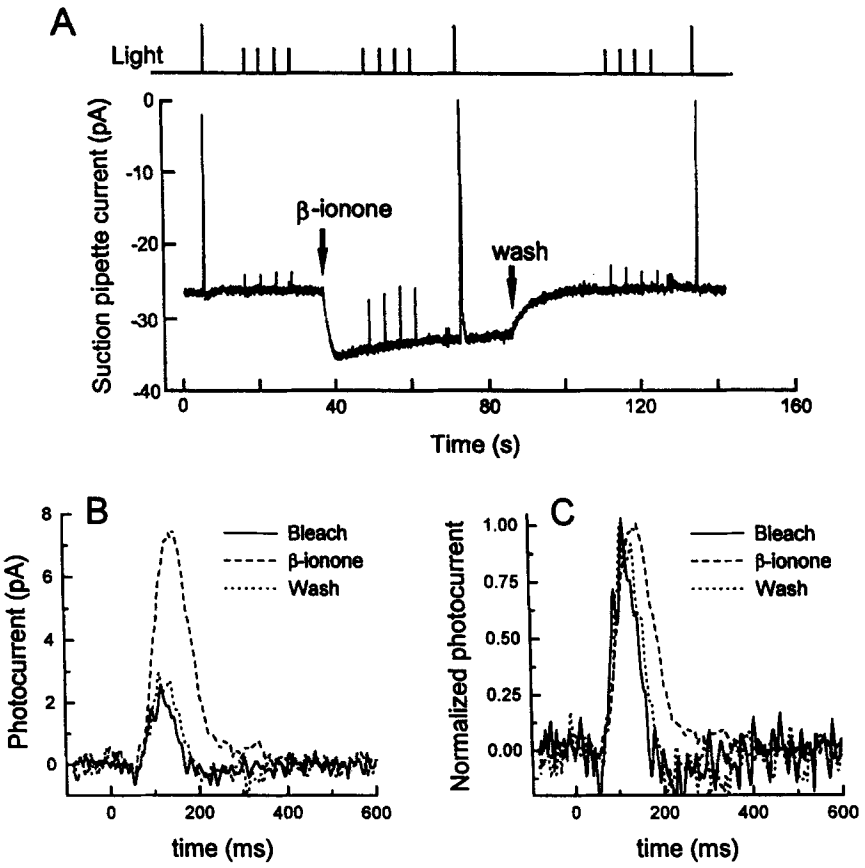

FIGURE 4. Rapid perfusion of bleached cone with β-ionone. Cone was initially exposed for 8 s to 600 nm light of intensity 3.4×10^7 photons μm^{-2} s^{-1}, calculated to bleach 82% of the photopigment. (*A*) Time course of β-ionone effect. Upper trace is stimulus marker, for which large upward deflections indicate flashes of intensity 3.3×10^6 photons μm^{-2}, and small deflections flashes of intensity 7.3×10^3 photons μm^{-2}. Flashes were at 600 nm and were 8.7 ms in duration. Exposure to β-ionone produced an increase in circulating current, accompanied by an augmentation in the amplitude of responses to both dim and bright flashes. These effects were rapidly reversed when the cone was moved out of the stream of the β-ionone back into control solution. (*B*) Effect of β-ionone on response waveform. Averages of four responses taken directly from records in *A* for bleached cone before β-ionone (*continuous line*), in β-ionone (*dashed line*), and after return to control solution (*dotted line*). (*C*) Same responses as in *B*, normalized to peak amplitude of 1.

in the current, which reflected an increase in the cone circulating current since it was accompanied by an increase in the size of the response to a saturating light flash. When the cone was stepped back into control solution, the circulating current decreased back to the previous bleached level.

Exposure to β-ionone also produced an increase in the amplitude of responses to dim flashes (Jin et al., 1993). Fig. 4 *B* plots on an expanded time scale averages of

responses recorded before perfusion with β-ionone, during perfusion, and after the return to control solution ("wash") for the cone of Fig. 4 *A*. The increase in response amplitude in β-ionone reflected a threefold increase in sensitivity. However, the sensitivity in β-ionone did not recover completely to its dark-adapted value, in part because β-ionone does not form a bleachable photopigment and therefore cannot restore desensitization resulting from loss in quantum catch (Jin et al., 1993). In six cones for which we have complete data, the sensitivity at 600 nm averaged $4.1 \times 10^{-2} \pm 1.8 \times 10^{-2}$ pA photon^{-1} μm^2 (or $1.8 \times 10^{-3} \pm 6.8 \times 10^{-4}$ [Rh*]$^{-1}$) before the bleach (means ± SE's), $4.7 \times 10^{-4} \pm 9.7 \times 10^{-5}$ pA photon^{-1} μm^2 (or $2.1 \times 10^{-5} \pm 3.3 \times 10^{-6}$ [Rh*]$^{-1}$) after the bleach, $1.5 \times 10^{-3} \pm 2.5 \times 10^{-4}$ pA photon^{-1} μm^2 (or $6.7 \times 10^{-5} \pm 6.3 \times 10^{-6}$ [Rh*]$^{-1}$) after exposure to β-ionone, and $4.4 \times 10^{-4} \pm 1.2 \times 10^{-4}$ pA photon^{-1} μm^2 (or $1.9 \times 10^{-5} \pm 3.6 \times 10^{-6}$ [Rh*]$^{-1}$) after return to control solution. The values in parentheses are the sensitivities given as fractional suppressions of maximal (dark) circulating current per photo-isomerization.

Responses in β-ionone-containing solution also decayed more slowly than in control solution after the bleach (Jin et al., 1993). This can be seen more clearly in Fig. 4 *C*, where the waveforms in Fig. 4 *B* have been normalized to their maximum amplitude. Return to control solution caused both the response amplitude and the time course of decay to return approximately to those observed before β-ionone treatment.

The results in Fig. 4. were typical of six cones rapidly exposed to β-ionone. In several cases, a single cone was exposed to β-ionone, returned to control solution, and then re-exposed to β-ionone two to three times during the same recording. For each exposure, results similar to those in Fig. 4 were obtained.

Effect of β-Ionone on Cyclase Velocity

Perfusion with β-ionone also altered the response of the cone to IBMX steps. In Fig. 5, a dark-adapted cone was first stepped into 500 μM IBMX, and the rate of current increase was measured (Fig. 5, *top*). We measured δJ_{max} to have been 1.3 s^{-1}. The cone was then exposed to light sufficient to bleach ~82% of the visual pigment, and the measured value of δJ_{max} increased to 6.0 s^{-1}. The cone was next stepped past the IBMX solution into the flow of solution containing 2 μM β-ionone. The cone was left in the β-ionone long enough for the circulating current and sensitivity to stabilize. It was then stepped into solution containing IBMX as well as β-ionone, and the rate of increase in circulating current was measured again. Under these conditions δJ_{max} decreased to 3.3 s^{-1}. Finally, the cone was returned to control solution, allowed to come to steady state, and stepped again into IBMX. The value of δJ_{max} increased back to 8.2 s^{-1}, somewhat more than its value in control solution just after the bleach.

In six similar experiments, δJ_{max} in darkness was 2.1 ± 0.3 s^{-1} (SE), increased to 7.1 ± 1.3 s^{-1} after an 82% bleach, returned to 3.3 ± 0.3 s^{-1} in β-ionone, and then accelerated back to 7.4 ± 0.8 s^{-1} after return to control solution. Following the bleach, the values of δJ_{max} before exposure to, and after return from β-ionone-containing solution were not statistically different ($p = 0.82$, *t* test). However, the value in β-ionone after the bleach was significantly larger than the value in control solu-

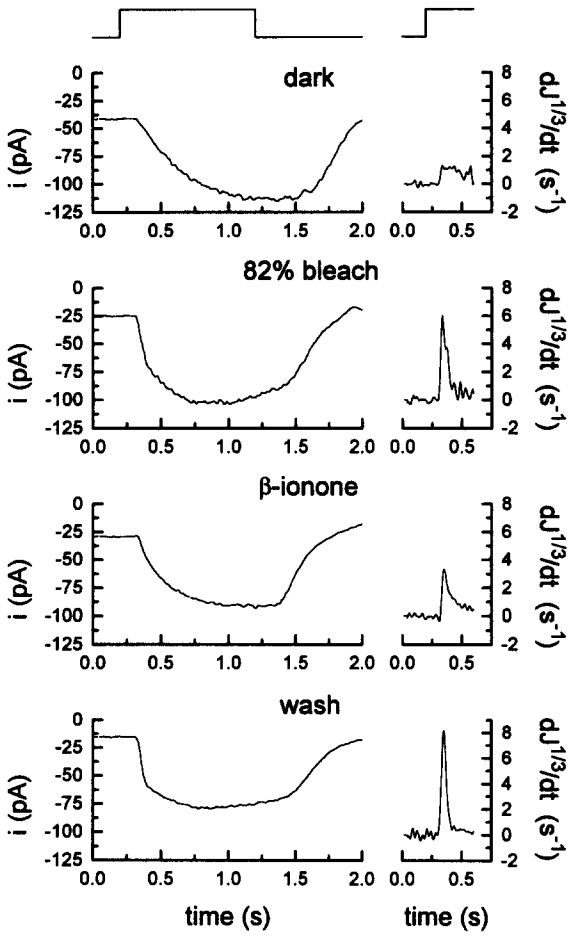

FIGURE 5. Effect of β-ionone on current responses of cone to steps into IBMX solution. (*Left*) Current responses to steps from control solution into 0.5 mM IBMX solution under the following conditions (top to bottom): in darkness; after exposure for 8 s to 600 nm light of intensity 3.4×10^7 photons $\mu m^{-2} s^{-1}$, calculated to bleach 82% of the photopigment; during exposure of bleached cone to β-ionone; and after return of bleached cone to control solution. (*Right*) Derivative of the one-third power of the normalized current for the first 0.6 s of the responses on the left. Each current trace is the average of four measurements. Uppermost traces give time course of triggers generated by PCLAMP software.

tion before bleaching ($P < 0.02$), suggesting that β-ionone (unlike 11-*cis*-retinal [see Fig. 2]) produced only a partial return of the cyclase rate. It is conceivable that greater recovery would have been observed had we used higher concentrations of β-ionone, but we did not attempt to do this since in our experience, exposure to higher concentrations tended to cause the circulating current and sensitivity of the cone to decrease irreversibly.

DISCUSSION

The evidence in this paper is consistent with the view that bleaching in a cone leads to an increase in the rate of the guanylyl cyclase. Because the guanylyl cyclase of photoreceptors appears not be modulated by light directly but rather indirectly via a decrease in intracellular free Ca^{2+} concentration, bleaching probably produces a maintained decrease in Ca^{2+} which is responsible for the cyclase acceleration we have observed (Matthews, Fain, and Cornwall, 1993, 1995).

In a photoreceptor at steady state, the rate of production of cGMP must be equal to its rate of destruction. An increase in the rate of the cyclase in backgrounds or after bleaches should therefore be accompanied by an increase in the rate of the

PDE. In rods it has been possible to demonstrate such an increase after bleaching (Cornwall and Fain, 1994) by measuring the rate of current decline after rapid steps into Li^+ solution (Hodgkin and Nunn, 1988). Similar experiments in cones were attempted as a part of this study but gave less satisfactory results. Although it was possible to demonstrate with bleaching a significant acceleration of the rate of current decline after stepping to Li^+, this acceleration was much smaller than predicted from the increase in cyclase rate (an 82% bleach increased β by only a factor of ~2). The reason for this, in our opinion, is that the velocity of the cone PDE is already so high in darkness (Perry and McNaughton, 1991) that we were unable to block the cyclase fast enough to observe the consequent speeding up of the PDE after bleaching.

Our experiments indicate that the increase in the cyclase velocity is nearly linear with percent pigment bleached but nonlinear with background intensity, over the same range of values of δJ_{max} and circulating current. The simplest explanation for this observation, in our view, is that light-activated pigment and bleached pigment have somewhat different effects on the transduction cascade (Cornwall and Fain, 1994). Both Rh* and bleached pigment appear to activate the PDE, decreasing the intracellular Ca^{2+} concentration (Matthews, Fain, and Cornwall, 1993, 1995) and increasing the rate of guanylyl cyclase. However, the decrease in Ca^{2+} may also modulate Rh* either directly (Lagnado and Baylor, 1994) or by activating rhodopsin kinase (Kawamura, 1993). A Ca^{2+}-dependent decrease in the activity or lifetime of Rh* would provide a further step in the feedback regulation of PDE and cyclase rates, which could be responsible for the nonlinear dependence of the steady state cyclase velocity with increasing background intensity (Fig. 3 *A*). Such a nonlinear dependence is not seen at steady state after bleaches (Fig. 3 *B*), perhaps because Ca^{2+} does not modulate the activity of bleached pigment. Other effects of Ca^{2+} (e.g. on the light-dependent channels, Hsu and Molday, 1993) may also play a role in bleaching or background adaptation.

We have shown that β-ionone can partially reverse the changes in circulating current, sensitivity, and response waveform produced by bleaching. The effects of β-ionone were more rapid in this study than previously reported (Jin et al., 1993), probably as a result of the faster method of perfusion. Beta-ionone can partially reverse the effect of bleaching on the rate of current increase in IBMX, which we interpret as an effect on the rate of the guanylyl cyclase. The effects of β-ionone are rapidly terminated upon return to control solution and can be observed repeatedly for the same cone. It would therefore appear that the chromophore pocket of the bleached intermediate under the conditions of our experiments is rapidly and reversibly accessible to β-ionone. The simplest explanation for this observation is that the chromophore pocket is empty (Corson, Cornwall, MacNichol, Jin, Johnson, Derguini, Crouch, and Nakanishi, 1990; Jin et al., 1993), and that the form of bleached pigment responsible for adaptation under the conditions of our experiments is opsin.

Our result suggest that opsin directly activates the transduction cascade and is responsible for the effect of bleaching at steady state on the cyclase velocity. The ratio of the effectiveness of opsin to that of Rh* can be calculated from

$$\frac{k_1/\delta J_{\max}^{D}}{(k_2/\delta J_{\max}^{D})\,t_i^{D}} \tag{8}$$

(see Cornwall and Fain, 1994), where t_i^D is the lifetime of the photoactivated pigment in a cone. The value t_i^D can be estimated from the integration time of cones in low-Ca^{2+}/zero-Na^+ solution and is ~0.4 s (Matthews et al., 1990). The values of $k_1/\delta J_{\max}^{D}$ and $k_2/\delta J_{\max}^{D}$ can be obtained from the fits of Eqs. 5 and 6.

We calculate the ratio of Rh* activation to opsin activation as about 6×10^4, which is about two orders of magnitude smaller than a similar ratio we have calculated for rods (Cornwall and Fain, 1994), even though $1/t_i^D$ is a factor of 10 larger. The ratio may be smaller for cones for two reasons: cones are less sensitive than rods, so that more Rh*'s are required to produce that same relative change in the cyclase ($k_1/\delta J_{\max}^{D}$ is smaller for cones); and cone opsin is more effective than rod opsin in activating transduction and increasing the cyclase velocity ($k_2/\delta J_{\max}^{D}$ is larger by a factor of ~20).

These calculations ignore possible differences in the effectiveness of phosphorylated and unphosphorylated opsin, or of opsin capped with arrestin. At present, virtually nothing is known about the molecular form of the pigment responsible for bleaching adaptation. This subject is of considerable interest, since different forms of opsin may play different roles in the mechanism of the generation of the equivalent background.

Our experiments suggest that bleaching adaptation may be produced by a similar mechanism in rods and cones. In both, bleached pigment activates the transduction cascade and produces an equivalent background that desensitizes the photoreceptor. The activation of the transduction cascade is approximately linear with the amount of pigment bleached, as if each opsin molecule in either a rod or a cone summed its effects linearly with all the other opsin molecules in that receptor. This could occur if any given opsin molecule, in whatever form it exists at steady state in the cone, were to activate the cascade with a probability that was independent of the number of opsins in the receptor. Bleaching activation can be reversed in both rods and cones when the photopigment is regenerated with 11-*cis* retinal, but partial reversal can be obtained (in cones) with β-ionone even without covalent attachment to the photopigment. Further experiments may reveal the role in bleaching adaptation of the other proteins in the transduction cascade (Matthews et al., 1994).

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